Salts Dramatically Enhance Activity of Enzymes Suspended in Organic Solvents

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Enzymes catalyze a diverse array of reactions in nonaqueous media.[1] However, their catalytic activities in organic solvents are often orders of magnitude lower than those in aqueous solutions. Recent studies have shown that the catalytic performance of enzymes in organic solvents can be significantly improved by the incorporation of carbohydrates,[2] polymers,[3] or organic buffers[4] into the dry catalyst, underscoring the important role of the enzymic microenvironment for catalysis in organic solvents. We report for the first time the dramatic enhancement of enzymatic activity in organic solvents brought about by the presence of simple nonbuffer salts in the lyophilized enzyme.

The data in Figure 1 and Table 1 show that the transesterification activity of subtilisin Carlsberg in anhydrous hexane[5] is strongly dependent on the KCl content of the lyophilized preparation and increases sharply when the salt content approaches 98%. For example, the value of k_{cat}/K_m for transesterification of *N*-Ac-L-Phe-OEt with 1-propanol, determined for the 98% KCl suspension (390 M^{-1} s^{-1}), is over 3750 times higher than that for the salt-free enzyme. Activation of subtilisin catalysis due to KCl occurs in a number of other widely different organic solvents, such as diisopropyl ether and tetrahydrofuran (Figure 1A), as well as acetonitrile, acetone, dioxane, and toluene (data not shown). The salt effect was not limited to subtilisin: the catalytic activity of α-chymotrypsin increased 50-fold when 95% (w/w) KCl was included in the lyophilized powder (Table 1). The improvement of catalytic activity for both enzymes appears to be primarily a result of a dramatically increased k_{cat} rather than a decreased K_m (Table 1). In addition to KCl, a number of other potassium salts were examined.[6] Every salt followed the trend depicted in Figure 1A—dramatic activation as the salt content approaches 95% in the biocatalyst powder. Importantly, the activation effect was absolutely dependent on the enzyme and salt being intricately associated with one another—no activation was observed if the same concentration of KCl was added to the reaction mixture containing salt-free enzyme powder.

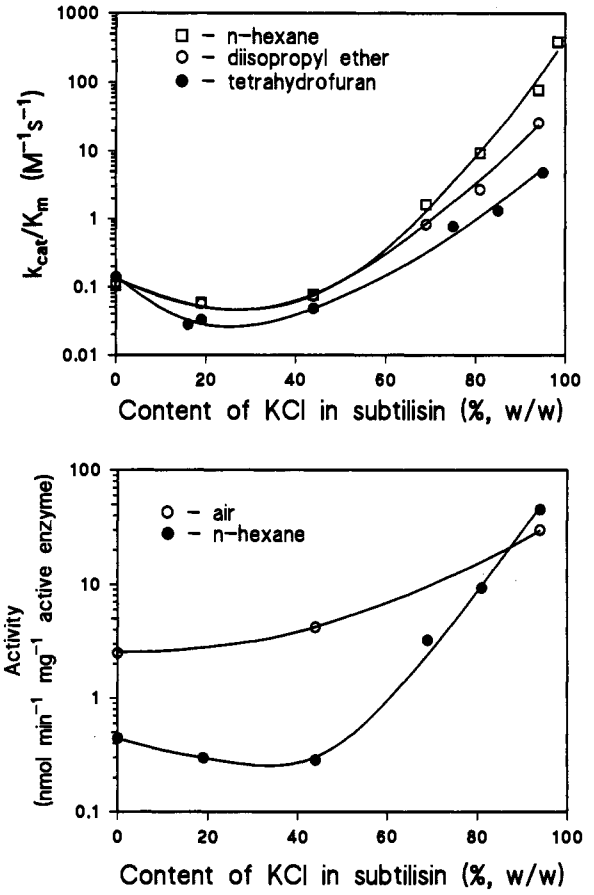

Figure 1. (A, top) Catalytic activity of subtilisin in anhydrous organic solvents as a function of the KCl content in the dry catalyst. The activity, expressed in terms of k_{cat}/K_m, was determined as described previously[14] using the transesterification reaction between *N*-acetyl-L-phenylalanine ethyl ester and *n*-propanol, used in concentrations of 10 mM and 0.85 M, respectively.[15] (B, bottom) Comparison of catalytic activities of subtilisin as a function of KCl content in the dry catalyst in liquid (*n*-hexane) and gaseous (air) media. The gas-phase transesterification reactions between 1-butanol and vinyl propionate were carried out at 30 °C in 28-mL glass vials sealed with a rubber septum. The reaction was initiated by injecting 5 mL of 1-butanol vapor and 2 mL of vinyl propionate vapor, saturated at 30 °C so that the concentrations of 1-butanol and vinyl propionate in the gas phase were 100 and 140 μM, respectively, into the reaction vial containing 10 mg of subtilisin. Periodically, gas samples were withdrawn with a gas-tight syringe and analyzed by gas chromatography (HP-1 capillary column). Concentrations of the substrates and product (butyl propionate) in the gas phase were determined from calibrations obtained by the GC analysis of standard vaporized samples. The reaction in *n*-hexane was performed at 30 °C and 250 rpm using concentrations of 1-butanol and vinyl propionate of 85 and 8 mM, respectively. The time course of the reaction was monitored using GC conditions similar to those described above for the gas-phase reaction.

Moreover, no change in activity was found when the concentration of phosphate in the KCl-based catalyst containing 5% enzyme was varied between 0 and 10%. Thus, catalytic activation was independent of the buffer properties of the enzyme–salt matrix. These findings indicate that the salt effect is neither solvent nor salt specific and reflects a general fundamental feature of the protein–salt matrix.

One could speculate that the observed increase in catalytic activity is the result of reduced internal diffusion limitations due to substantial dilution of the biocatalyst in the enzyme–salt composite. However, conservative estimates of the observable modulus, Φ, indicate that the actual situation is reversed, i.e., it is the salt-rich subtilisin powder that is more likely to be affected by internal diffusion, whereas catalysis by the salt-free enzyme

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(5) Enzyme samples were prepared by lyophilizing different amounts of enzyme, salt, and potassium phosphate buffer from an aqueous solution (pH 7.8) for 48 h. Unless indicated otherwise, the buffer content in the final preparation was 1%, w/w. Lyophilized samples were stored over molecular sieves. All chemicals and solvents used in this work were of the highest grade commercially available. All solvents were stored over molecular sieves at least 24 h prior to use.

(6) The following potassium salts (along with their k_{cat}/K_m values in M^{-1} s^{-1}, and activation factor over enzyme preparation without salt added) were employed: Cl (78.6, 756); acetate (27.2, 262); NO_3 (25.8, 248); SO_4 (18.9, 182); and F (18.1, 174).

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Table 1. Effect of KCl as a Salt Matrix on Subtilisin Carlsberg and Chymotrypsin in Anhydrous Hexane[a]

enzyme	salt content (%, w/w)	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m (M^{-1} s^{-1})
subtilisin	0	0.027	260	0.104
	98	10.4	26.7	390
chymotrypsin[b]	0	4.2×10^{-4}	33.0	0.013
	94	220×10^{-4}	33.0	0.67

[a] For the transesterification reaction of *N*-Ac-L-Phe-OEt with 0.85 M 1-propanol. [b] The concentration of active sites of α-chymotrypsin in *n*-hexane was assumed to be approximately equal to that found in aqueous solutions (66% of the total protein).[13] It should be noted that even if the active fraction of active sites for chymotrypsin in the nearly pure enzyme suspension was only 10% (the same as for subtilisin) and the fraction of active sites in the salt-rich catalyst was 66%, only a 6-fold enhancement in k_{cat} (and thus k_{cat}/K_m) would result.

powder is practically free of diffusional limitations.[7] Thus, the intrinsic rate enhancement may be even somewhat *higher* than the observed activation. Furthermore, previous kinetic studies[8] have shown that the activity in anhydrous organic solvents of subtilisin powders containing low amounts of buffer salts (up to 35%, w/w) was unaffected by internal diffusion.

The mechanism of salt-induced activation of enzyme activity in organic solvents may be due to at least two critical factors: the salt may protect the enzyme from direct inactivation by the organic solvent, or the salt may help to maintain the enzyme's native structure during lyophilization. This latter phenomenon has been suggested to be dominant in the observed activation of enzymes that are prepared with sugars[2b] or polymers such as polyethylene glycol[9] as lyoprotectants. In order to investigate the importance of these phenomena, activities of subtilisin/KCl catalysts were compared in both a liquid and a gaseous medium; the latter presumably is inert toward enzyme molecules relative to the organic solvent. Hexane was chosen as a representative liquid medium, while air was used as the gaseous medium. Transesterification reactions of vinyl propionate with 1-butanol were performed. These substrates are soluble in hexane and have sufficiently high vapor pressures to be present in the gas phase at 30 °C. As depicted in Figure 1B, the presence of increasing amounts of KCl in the enzyme powder dramatically increases the activity of subtilisin in hexane—the enhancement upon adding 95% (w/w) KCl to the lyophilized powder was over 100-fold (an increase consistent with that obtained with *N*-Ac-L-Phe-OEt for 95% (w/w) KCl; compare Figures 1A and 1B). In contrast, in the case of the gas-phase reaction, the enhancement was less than 10-fold. If enzyme activation was due to lyophilization, then the observed magnitude of the rate enhancement should be the same in both media. Thus, the activation phenomenon observed in organic solvents cannot be explained solely in terms of a lyoprotectant effect of salts.

We hypothesize that the observed activation upon entrainment of the enzyme in a salt matrix is due to a protective effect afforded by the matrix against deactivation by direct contact with the organic solvent. At low salt contents, the solid catalyst particles are composed primarily of the lyophilized protein which forms a loosely structured environment.[10] At higher salt contents, the catalyst particles presumably are more like that of free salt, which has a rigid structure that may protect the enzyme from the organic solvent. Furthermore, the salt matrix is highly polar and may help to maintain the native structure of the enzyme in organic media.[11] We are now in the process of using structural and polarity probes to test these hypotheses.

The salt effect may have important practical implications and should lead to an improved understanding of enzyme function in organic solvents. Our results show that not only the pH of the starting aqueous solution is important to the activity of the final biocatalyst,[12] but the enzyme/buffer salt ratio is also a critical factor. The influence of this ratio, noticed in several earlier studies,[13] can now be explained in terms of the salt matrix effect.

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(7) For spherical geometry, the observable modulus, Φ, is defined as

$$\Phi = \left(\frac{R}{3}\right)^2 \frac{v_{obs}}{D_{eff} S_0}$$

where v_{obs} is the measured reaction rate per volume of catalyst, D_{eff} is the effective diffusivity of substrate, S_0 is the bulk-phase substrate concentration, and R is the particle radius. The modulus is a measure of the extent to which internal diffusion limits the overall reaction rate. Assuming $D_{eff} = 10^{-6}$ cm^2 s^{-1} and $R = 50$ μm gives Φ values of 3.5 (moderate diffusional limitations) and 0.88 (slight to no diffusional limitations) for subtilisin lyophilized with and without salt, respectively. Although D_{eff} may differ for the two preparations, for both cases 10^{-6} cm^2 s^{-1} is a conservative estimate.

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(15) The percentage of catalytically active centers in organic solvents was taken as 10 ± 5% of the total protein based on the results of direct titrations in tetrahydrofuran.[14] The value of k_{cat}/K_m was calculated from initial rate data ($v/[S]$), as the concentration of *N*-Ac-L-Phe-OEt was appreciably below the K_m value.